Exhibit E

3 - 2

Ability of the Sonicare® Electronic Toothbrush to Generate Dynamic Fluid Activity that Removes Bacteria

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Abstract

Two laboratory studies were performed to investigate removal of bacteria adherent to model oral surfaces by a new electronic toothbrush, the Sonicare®. Sonicare produces dynamic fluid activity and mild cavitation due to the high frequency vibration of its bristles. The purpose of the study was to determine if this fluid agitation alone could dislodge bacteria from oral surfaces. Commercially pure titanium disks were ground to a finish comparable to dental implants and coated with either *Streptococcus mutans* or *Porphyromonas gingivalis* as a model for bacterial plaque on implants. Additionally, saliva-coated hydroxyapatite disks were coated with *Actinomyces naeslundii* as a model for bacterial plaque on a tooth surface. Electron and fluorescence microscopy were utilized to quantitate bacterial adherence before and after exposure to Sonicare-induced fluid forces. Bristle tip to model surface distances of 0, 1, 2, 3, and 4 mm were investigated. It was found that after 15 seconds of exposure with direct bristle contact, the reduction in adherent bacteria was nearly 100% for all 3 species, with an average 75% reduction at a distance of 2 mm. At a 4 mm distance, the reductions were 61% for *S. mutans* and 24% for *A. naeslundii*, with a minor dislodgment of *P. gingivalis*. The results indicate that the Sonicare is able to remove bacteria from these model dental surfaces due to fluid dynamic action which extends up to 4 mm beyond the reach of the bristles. **(J Clin Dent 5:89–93, 1994.)**

Introduction

Standard toothbrushing relies on direct contact between the toothbrush bristles and the tooth surface to remove dental plaque. The user of a manual toothbrush places the bristles against the teeth and physically moves the bristles to scrub plaque from the surfaces the bristles touch. Powered toothbrushes move the bristles across the tooth to remove plaque while relying on the user to position the toothbrush about the dentition. Other instruments such as proxibrushes, stimudents, and floss also rely on direct contact with the plaque accumulations to clean the tooth surface. As an alternative to direct bristle contact, oral irrigators are meant to remove plaque from teeth via fluid forces. Irrigators project a high velocity fluid jet that may be directed into areas where the bristles of a toothbrush cannot reach. Although both types of devices, direct contact or irrigation, possess particular strengths, most studies have found neither device to be totally effective by itself in removing plaque accumulation on all surfaces.[1,2]

A combination of direct mechanical scrubbing and penetrating fluid motion can be found in a new powered toothbrush that uses high frequency bristle motion to create dynamic fluid activity. Utilizing research originating at the University of Washington (Seattle, WA), Optiva Corp. (Bellevue, WA) developed the Sonicare® electronic toothbrush. The bristles of the Sonicare vibrate at 260 Hz, creating mild cavitation and bubble activity about the Sonicare brush head. Previous studies have shown that sonic vibrations in fluid similar to those generated by Sonicare can alter cell surface structures of the oral bacterium *Actinomyces naeslundii* (formerly *Actinomyces viscosus* T14V) as well as remove these adherent bacteria from model dental surfaces *in vitro*.[3,4] A previous clinical study of Sonicare's efficacy demonstrated its superiority over the manual toothbrush in removing supragingival plaque, particularly in hard to reach areas such as the interproximal regions and the posterior teeth.[5]

The purpose of the current study was to investigate the ability of Sonicare to remove bacteria from model dental surfaces without direct bristle contact, i.e., whether the fluid forces alone can remove adherent oral bacteria. Two independent studies were conducted at separate sites: 1) bacterial adherence to implant surfaces was modeled using titanium disks with adherent *Streptococcus mutans* or *Porphyromonas gingivalis*; and 2) bacterial adherence to the tooth surface was modeled using saliva-coated hydroxyapatite (SHA) with adherent *A. naeslundii* (Optiva Corp.).

Materials and Methods

Bacteria

S. mutans (strain Ingbritt) were grown in brain-heart infusion broth (BHI, Difco, Detroit, MI) with 2% sucrose for 48 hrs at 37°C. *P. gingivalis* (ATCC strain 33277) were routinely grown in trypticase soy broth (TSB) containing L-cysteine hydrochloride (0.5 g/l), yeast extract (5 g/l), hemin (5 µg/l) and menadione (0.2 µg/l) (PG broth) for 48–72 hrs at 37°C anaerobically

in an atmosphere of 10% H_2, 5% CO_2, and 85% N_2. *A. naeslundii* (strain T14V) were grown in TSB supplemented with 0.1% yeast extract and 0.5% glucose for 16 hr at 37°C with continuous agitation. *A. naeslundii* were prepared for adherence assays as previously described.[3]

Adherence Models

To prepare model implant surfaces, methods described by Keller *et al.* were used.[6] Titanium (Ti) disks (12.5 mm diameter, 3.5 mm thick) obtained from commercially pure Ti-based bar stock (Alfa Products, Danvers, MA) were hand ground using a 1 µm grit diamond polishing paste to produce a final surface finish comparable to commercially available implants. For disk orientation purposes during exposure and examination, two parallel lines were scribed 3 mm from opposite edges of the disk. The disks were cleaned using methylethyl ketone (5 min), rinsed with ultra-pure water (15 min), acid-passivated with 30% nitric acid (30 min) and finally rinsed with ultra-pure water (20 min).

Chambers for adherence of the bacteria on the Ti surface were prepared as described previously.[7] Tygon tubing (12.7 mm ID, 1.6 mm wall thickness, 30 mm length) was cleaned with RBS 35, 2% (Pierce Chemicals, Rockford, IL) for 5 min, rinsed 15 min with tap water, agitated 2 min in Prosil 28, 1% dilution (PCR Inc., Gainesville, FL) and rinsed 30 min with distilled water.[8] The tubing was fitted over the Ti disk and the bottom sealed with Regisil bite registration material (L.D. Caulk Div., Dentsply Intl., Milford, DE) to achieve a fluid-tight adherence chamber. The chambers were sterilized 30 min with UV light (300 µw/cm^2) prior to incubation with the bacteria.

Ti disks (n = 12) were pre-colonized with *S. mutans* by growing the bacteria for 48 hrs in BHI-2% sucrose medium in the chambers described above. At the end of the incubation period, the culture medium was removed. The Ti surface with attached *S. mutans* was carefully rinsed with sterile phosphate buffered saline (PBS, 0.05M, pH 7.0) prior to exposure to Sonicare. Additionally, separate Ti disks (n = 12) were coated with *P. gingivalis* by a previously established method.[7] For these, the Ti disks were pre-coated with 0.25 ml 10% fetal calf serum at 37°C for 1 hr and the disk surface washed 3 times with PBS. A 0.5 ml aliquot of *P. gingivalis* (5 x 10^6 cells/ml in PG broth) was placed onto the serum-coated disks and incubated anaerobically for 1 hr at 37°C. After incubation, the non-attached cells and suspending medium were carefully aspirated from the chamber and the disk surfaces washed 3 times with PBS prior to exposure to the Sonicare.

For preparing *A. naeslundii*-coated SHA disks, procedures previously established were used.[3] Hydroxyapatite disks 5.2 mm diameter, 2.4 mm thick (Calcitek, Inc., Carlsbad, CA) were cleaned in an ultrasonic water bath, rinsed in KCl buffer[8] (0.05M pH 6.0) for 1 hr, and then coated with saliva overnight. Freshly-harvested *A. naeslundii* were washed 3 times in carbonate buffer (0.1 M, pH 9.6), incubated with fluorescein isothiocyanate isomer I (FITC, 0.25 mg/ml in carbonate buffer) for 2 hr, and washed 3 times in KCl buffer. The FITC-labelled *A. naeslundii* were then added onto SHA and further incubated at room temperature for 1 hr with agitation. The disks were rinsed in KCl buffer to remove non-adherent bacteria.

Exposure to the Sonicare

For Sonicare exposure, a bacteria-coated disk was rigidly mounted in a holding chamber below the Sonicare so that the peak of the tuft of bristles furthest out from the brush handle was mounted centrally over the disk, as shown in Figure 1. Ti disks were oriented so that the activated Sonicare bristles, moving at 260 Hz (520 strokes/sec), swept parallel to the scored lines on the disk. The bristle-to-surface distance was set to either 0 (contact), 1, 2, 3, or 4 mm. PBS was placed in the holding chamber so that the fluid level reached 2 mm into the tips of the shorter Sonicare bristles. The Sonicare was then activated and moved across the disk surface, ±3 mm from the central position in a direction perpendicular to bristle movement, approximately twice every 5 sec. The exposure times were between 5 and 30 sec. Control disks were not exposed to the Sonicare, but were mounted in the holding chamber for 30 sec in a manner similar to the exposed disks. All brushings were done with a fully charged Sonicare power handle.

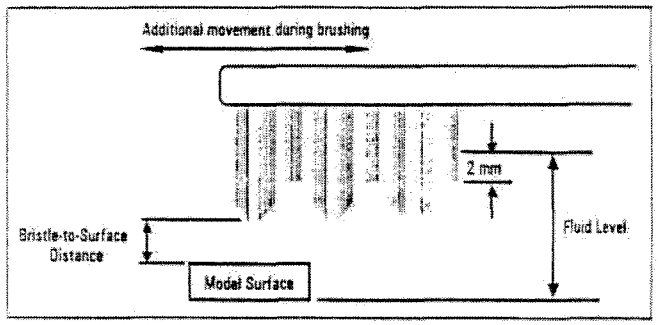

Figure 1. *The model dental surface was positioned below the Sonicare bristles and covered with fluid to a consistent level extending into the bristles. The bristle-to-surface distance was altered to investigate the ability of the Sonicare to remove bacteria from surfaces beyond the reach of the bristles. Normal Sonicare bristle movement is in and out of the page. To evenly expose the surfaces, the Sonicare was additionally moved by hand in the direction shown.*

After exposure, the disks were removed, gently rinsed with PBS or KCl buffer, and further processed for scanning electron microscopy (SEM) or fluorescent microscopy. Triplicate disks of each exposure condition for each respective bacteria were performed for the Ti study. Eight replicates of each exposure condition were performed for the SHA study. Throughout the studies, handling of the model surfaces was minimized to prevent inadvertent removal of bacteria.

Examination of the Disk Surfaces with SEM or Fluorescent Microscopy

Both control Ti disks and disks exposed to the Sonicare were rinsed twice with cacodylate buffer (CD buffer, 0.1 M, pH 7.0), placed in vials containing 3% glutaraldehyde in CD buffer (1 hr), washed with CD buffer, and dehydrated through a graded acetone series (30%–100%). After critical-point drying (model CPD020, Balzers Union Ltd., Liechtenstein), the disks were sputter coated with gold-palladium alloy (model SCD040, Balzers Union) and viewed with a scanning electron microscope (Hitachi S4000).

For each disk specimen, SEM micrographs (2000×) were taken over three designated fields: one centrally located on the

disk and two near the ends between the scribed lines. The bacterial counts for all three micrographs were enumerated and expressed in bacteria per mm^2. The mean was obtained from triplicate specimens. For *S. mutans* colonized control specimens, SEM micrographs often demonstrated multilayered bacterial aggregates. An approximation of the bacteria on the surface was made based on the number of bacteria found in a monolayer multiplied by an estimation of the number of layers of bacteria. An ANOVA and Student-Newman-Keuls Multicomparison Tests were used for statistical analysis.

Fluorescence microscopy was used to quantitate the number of *A. naeslundii* on SHA surfaces. A silicon photodetector with digital power meter (models 818SL and 815, Newport Corp., Irvine, CA) was mounted in the camera tube of an optical microscope. A bacteria-coated SHA disk was placed under a 4× objective and illuminated at 490 nm. Light emitted by the fluorescing dye on the bacteria was measured with the photodetector. Disks with no adherent bacteria were used to determine that the average background reading due to light reflection was 40 μW; therefore this value was subtracted out of all subsequent readings of the bacteria-coated disks. The fluorescence of the bacteria on each experimental disk was measured immediately before and after exposure to the Sonicare. The difference in fluorescence was used as an indicator of bacterial removal. Statistical testing of the reduction in photometer readings was completed with an ANOVA along with Student-Newman-Keuls Multicomparison Tests to investigate differences from the control.

Examination of Titanium Surface after Direct Brushing

Six Ti disks, prepared as described above, except without attachment of bacteria, were used to establish the effects of the Sonicare on polished Ti. Two disks were used as controls and received no brushing. Four disks were submerged under 2 mm distilled water and brushed continuously with the Sonicare with direct bristle contact for the equivalent of 3 months normal use (approximating 8 sec brushing per day). The disks were processed for SEM examination. SEM micrographs taken of representative surface areas of both the control and exposed Ti disks (500–20,000 X) were examined for differences in morphology.

Results

Although not shown here, preliminary studies were performed to determine the optimal condition for bacterial colonization of Ti surfaces. Inoculation of *S. mutans* onto Ti disks of the adherence chamber, in the presence of sucrose-containing BHI medium for 24 hr at 37°C allowed the formation of a firmly attached bacterial mass on the Ti disk (Figure 2A). Bacteria were often observed in multilayers or in clusters and were not removable by rinsing with PBS.

P. gingivalis did not attach firmly to Ti disk surfaces when grown in PG broth up to 72 hr at 37°C under anaerobic conditions (data not shown). By following the method of Wu-Yuan *et al.*,[7] it was possible to obtain a Ti surface uniformly covered with *P. gingivalis* as revealed by SEM (Figure 2E). This allowed the enumeration of adherent bacteria on both control and exposed Ti surfaces.

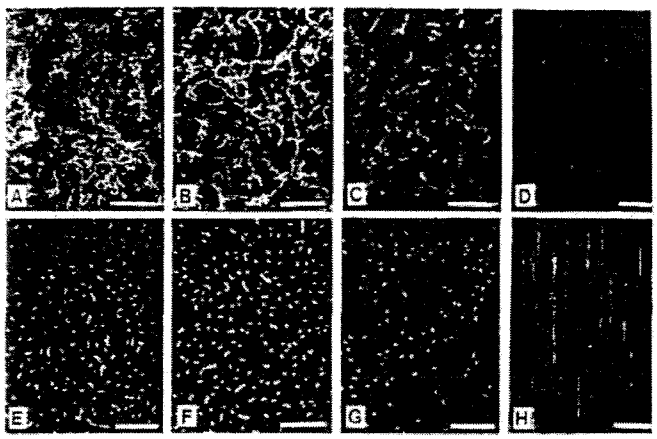

Figure 2. *Bacteria-coated Ti surfaces before and after exposure to Sonicare: S. mutans—panels A, B, C, D; P. gingivalis—panels E, F, G, H. Control surfaces (A&E) exhibit adherent bacteria without exposure to Sonicare. Fewer bacteria remain adherent after 15 seconds of exposure at bristle-to-surface distances of 4 mm (B&F), 2 mm (C&G) and 0 mm (D&H). Bars: 10 μm.*

SEM micrographs taken of the Ti surfaces before and after 15 sec of Sonicare exposure are shown in Figure 2. At a bristle-to-surface distance of 4 mm, there is an obvious reduction in the number of *S. mutans* remaining adherent (Figure 2B), but less so for the *P. gingivalis* (Figure 2F). At a closer distance of 2 mm, there is further removal of *S. mutans* (Figure 2C) and an evident removal of *P. gingivalis* (Figure 2G). When the bristles directly contacted the surface, nearly all the *S. mutans* were removed (Figure 2D) while no adherent *P. gingivalis* were noted (Figure 2H).

The numbers of bacteria adherent to the Ti disks before and after exposure to Sonicare were enumerated and are presented in Table I. The mean value presented in the table represents the average of 9 counts (3 counts of 3 disks for each condition). The control condition, i.e., no Sonicare exposure, is given in the first row of the table with subsequent rows representing increasing proximity of the Sonicare bristles to the disk surface during 15 sec of exposure. A one-way ANOVA indicated a significant difference ($p = 0.0008$ for *S. mutans*, $p = 0.0002$ for *P. gingivalis*) for the exposure conditions. Analysis with *post hoc* t-tests demonstrated a significant reduction ($p < 0.05$) from the unexposed control for all conditions except *P. gingivalis* at 4 mm.

Table I
Dislodgment of *S. mutans* and *P. gingivalis* from Ti surfaces

Exposure (15 sec)	*S. mutans*, 10^3 cells/mm^2 mean[a] ± se	*P. gingivalis*, 10^3 cells/mm^2 mean[a] ± se
Control	1860 ± 390	154 ± 19
4 mm	729 ± 36[b]	149 ± 17
2 mm	300 ± 39[b]	60 ± 15[b]
0 mm	7 ± 5[b]	0 ± 0[b]

[a]Mean value obtained from 9 counts (3 counts each of triplicate disks).
[b]Significantly different from control ($p < 0.05$).

Photometric readings of the light emission of FITC-labeled *A. naeslundii* provided a rapid and repeatable method of determining the quantity of bacteria adherent to the SHA disks. The average photometric reading of bacterial-coated disks prior to Sonicare exposure was 383 μW with a standard error of 7.5 μW.

The control conditions, in which the disks underwent handling and placement below the inactivated Sonicare, resulted in a 4.0 ± 0.5% reduction in fluorescence. Table II shows the reduction in adherent bacteria as calculated from the change in photometric readings and with the control reduction subtracted out. As the values in the table demonstrate, a greater distance between the bristles and the surface resulted in the removal of fewer bacteria. Additionally, slightly fewer bacteria were removed with a shorter exposure duration, however the majority of the bacteria that were to be removed were done so within the first 5 sec of exposure. A significant difference ($p < 0.0001$) for the exposure conditions was shown with a one-way ANOVA. *Post hoc* t-tests indicated all reductions were significantly different ($p < 0.05$) than the unexposed control, except for 5 sec at 4 mm.

Table II
Dislodgment of *A. naeslundii* from SHA surfaces

Exposure Condition	Percentage Reduction in Bacteria (mean[a] ± se)		
	5 sec	15 sec	30 sec
1 mm	83 ± 3[b]	87 ± 2[b]	90 ± 1[b]
2 mm	74 ± 2[b]	81 ± 1[b]	85 ± 2[b]
3 mm	54 ± 7[b]	79 ± 2[b]	79 ± 2[b]
4 mm	13 ± 5	24 ± 8[b]	18 ± 10[b]

[a]Mean value obtained from 8 SHA surfaces.
[b]Significantly different from control ($p < 0.05$).

Figure 3 graphically demonstrates the percentage reduction of adherent bacteria from dental surfaces after Sonicare exposure based on the data presented in Tables I and II. The reduction in adherent bacteria was obtained by comparing the numbers of bacteria remaining on Ti disks after exposure to numbers present on the non-exposed disks. There was virtually a 100% reduction in adherent bacteria with bristle contact (0 mm). At a bristle-to-surface distance of 2 mm, the reduction in adherent bacteria was 84% for *S. mutans*, 81% for *A. naeslundii* and 61% for *P. gingivalis*. There was greater variation in the results at 4 mm, with reductions of 61% for *S. mutans*, 24% for *A. naeslundii*, and few *P. gingivalis* being dislodged.

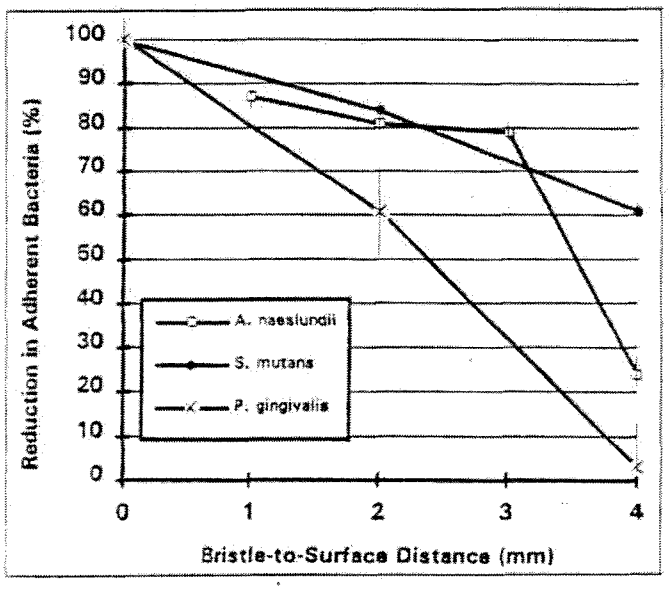

Figure 3. *Reduction in the number of adherent bacteria from model dental surfaces after 15 sec exposure to the Sonicare. The percentage reduction due to Sonicare exposure was calculated as described in the text. Vertical bars indicate the average standard error for each condition.*

Although not presented here, no abrasion of the Ti surface due to direct contact with the Sonicare bristles was revealed when examined with SEM. Both the exposed and non-exposed Ti surfaces, when examined under SEM, appeared smooth with occasional groves created by the polishing procedures.

Discussion

Removal of adherent bacteria associated with dental plaque is important in maintaining the health of the oral cavity. The presence of dental plaque results in tissue irritation that leads to gingivitis, and potentially to periodontal disease or peri-implantitis. Clinical and microbiological findings of healthy and failing dental implants have suggested that bacteria implicated as pathogens in periodontal disease may play a role in the failure of implants.[9-11]

Bacterial adherence and colonization are considered key factors in the etiology of dental plaque and biomaterial-based infections.[12,13] *S. mutans* has been strongly implicated as the etiological agent of dental caries[14] and is involved in early dental plaque formation, as is *A. naeslundii*.[15] *P. gingivalis* is a periodontopathic bacterium that has been associated with periodontal disease.[16] Although the bacteria studied here do not fully represent the complex interbacterial relationships that are involved in dental plaque formation, they are representative of important mechanisms involved in bacterial adherence to dental surfaces.

Many individuals do not adequately remove sufficient dental plaque to maintain healthy teeth and gingival tissue with the use of either manual or conventionally powered toothbrushes. Toothbrushes are limited in their cleaning ability by the requirement of contact between dental plaque and the toothbrush bristles. Although oral irrigators can potentially reach areas toothbrush bristles do not, accurately aiming the water jet on all tooth surface, is difficult and their efficacy in plaque removal remains doubtful.[1,17] An advantage of the powered toothbrush studied here is that it combines both direct mechanical bristle scrubbing with fluid activity and mild cavitation that extends into areas the bristles do not reach.

The results from this study indicate that the Sonicare creates sufficient fluid activity to remove bacteria without direct bristle contact. Significant bacterial removal was observed at distances up to 4 mm from the bristles (Figure 3). Nearly all adherent bacteria were removed from Ti or SHA surfaces with complete bristle contact or at a distance very close to the exposed surface (Tables I and II). In the oral cavity the greatest buccolingual distance not reachable by toothbrush bristles from either the lingual or buccal side is on average 3 to 4 mm. Removal of bacteria adherent in these areas due to the fluid motion of the Sonicare may compensate for the limitation of contacting all areas with bristles.

The removal of bacteria via dynamic fluid activity at distances beyond 2 mm appears to vary with the characteristics of the adherent bacteria. As evident from Figure 2, exposure of *S. mutans*-colonized Ti disks to Sonicare at 2 and 4 mm reduced

the number of adherent bacteria projecting from, but not directly attached to the surface. This is consistent with the SEM observations of *A. naeslundii* after exposure to sonic vibrations, as previously reported.[4] Bacteria projecting from the surface are at greater risk of being subjected to shear forces from the fluid motion, thus are likely the first to be removed. Bacteria closer and more tightly adherent to the surface may require a longer exposure period or a closer bristle proximity to be effectively removed. In contrast to *S. mutans* which adhered in clusters, *P. gingivalis* appeared to adhere individually to Ti (Figure 2E). It is possible that because the bacteria do not project far beyond the Ti surface, a closer proximity of the bristles to the surface may be required for their removal (Figures 2F and 2G).

In the oral cavity, plaque forms complex interbacterial associations projecting beyond the dental surfaces. With regular Sonicare use, bacteria protruding from a surface may be removed in sufficient quantities to prevent significant additional bacterial accumulation. However, the dynamics of brushing in the oral cavity will be different than an *in vitro* situation, i.e., saliva and toothpaste may alter the fluid dynamics, fluid quantities around the brush head may vary, and bacterial inter-relationships and adherence may be more complex.

In summary, the studies presented here have shown that shear forces associated with mild cavitation and fluid activity surrounding the Sonicare head as it oscillates at 260 Hz are sufficient to remove bacteria adherent to model dental surfaces. Such activity, when combined with direct mechanical scrubbing of the high frequency Sonicare bristle movement, may effectively remove bacteria both on oral surfaces such as teeth and implants and in hard to reach regions of the oral cavity.

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